1 The role of CFTR genetic testing in diagnosis of cystic fibrosis

S. Fustik[1], T. Jakovska[1]. [1]*University Pediatric Clinic, Center for Cystic Fibrosis, Skopje, Macedonia, the Former Yugoslav Republic of*

The large number of different mutations in CFTR gene combined with marked variation in their distribution and frequency make the genetic diagnosis of CF rather difficult. The aim of the study was to estimate the detection rate of CFTR mutations in our patients according to the results from the genetic testing performed at Research Center for Genetic Engineering and Biotechnology (Skopje).

Methods: CFTR genotype results from 140 patients who had confirmed clinical diagnosis of CF were analyzed. The mainly used molecular analysis methods included: PAGE of PCR-amplified fragments of DNA for the detection of F508del; Inno-LiPA CFTR17 and Inno-LiPA CFTR19 panels for identification of 36 CFTR mutations; and DGGE mutations screening, followed by sequencing of samples showing an abnormal pattern.

Results: Mutation detection rate in our group of patients was 83%. The frequency of the most common mutations was: F508del (69.2%), G542X (6.1%), N1303K (2.9%), 621+1G>T (1.1%) and CFTRdel2,1(21kb) (1.1%). Six DNA samples from CF patients in which one or no CFTR mutations have been identified were sent for analysis in Laboratoire de Génétique Moléculaire, Brest (France). With more extensive screening/sequencing protocols and methods for identification of large rearrangements, all unknown mutations were identified.

Conclusion: The diagnosis of CF remains clinical. Indetermination of CFTR mutations arouse doubts in families about the accuracy of diagnosis. Because of genetic heterogeneity of CF in our region, there is a need for additional methods for CFTR gene analysis to maximize mutant allele detection rate, which will have positive implications for the genetic counseling of families.

2 The syndrome of exocrine pancreatic insufficiency – cystic fibrosis or other disease?

A. Sobczyńska-Tomaszewska[1,2], K. Czerska[1,2], A. Szpecht-Potocka[1], D. Popielarz[1], K. Wertheim-Tysarowska[2], K. Niepokój[2], B. Oralewska[3], J. Bal[2]. [1]*Health Care Center Genomed, Warsaw, Poland;* [2]*Institute of Mother and Child, Department of Medical Genetics, Warsaw, Poland;* [3]*Children's Memorial Health Institute, Department of Gastroenterology, Warsaw, Poland*

The Shwachman-Diamond syndrome (SDS), is rare disease (1/100 000 live births) caused by compound heterozygous/homozygous mutations in the *SBDS* gene. SDS is characterized primarily by exocrine pancreatic insufficiency, hematologic abnormalities, including increased risk of malignant transformation, and skeletal abnormalities. The pancreatic insufficiency which is observed in this syndrome, may suggests clinical observation for cystic fibrosis and may caused in delay in diagnosis of patient. A Polish girl with abnormal result of IRT during newborn screening programme for cystic fibrosis was directed for clinical and genetic evaluation for cystic fibrosis and *CFTR* gene analysis. Sequencing of whole coding region of the *CFTR* gene together with identification of frequent for Polish population mutations: dele2,3(21kb) and 3849+10kbC>T were performed. No pathogenic *CFTR* variants were identified. Only the I148T variant, without clinical consequence according to CF recommedation was identified. Because patient presented chronic pancreatic and liver disease, anemia, decrease concentration of stool elastase, hipertransaminasemia, borderline/normal sweat test results and short stature were observed, clinical observation and genetic analysis for Shwachman-Diamond syndrome was suggested as a next diagnostic stage. Analysis of coding region of the *SBDS* gene were performed. Two known mutations: c.258+2T and p.C119Y, each in one *SBDS* allele were identified. Biparental inheritance of identified mutations was confirmed. In conclusion, because of the similarity of some symptoms in cases of atypical abdominal form of the CF, the SDS should be taken into account during diagnosis process.

3 *CFTR* gene direct sequencing *versus* scanning techniques. Improving the sensitivity to identify CF mutations

D. Trujillano[1], M.D. Ramos[2], A. Vazquez[2], X. Estivill[1], J. Manzanares[3], T. Casals[2,4]. [1]*Centre for Genomic Regulation, Genes and Disease Program, Barcelona, Spain;* [2]*IDIBELL, Center for Molecular Genetic Diagnosis, Barcelona, Spain;* [3]*Hospital 12 de Octubre, Cystic Fibrosis Unit, Madrid, Spain;* [4]*IDIBELL, Human Molecular Genetics Group, Barcelona, Spain*

From the *CFTR* gene cloning, the scanning techniques (SSCP/HD, DGGE, DHPLC, HRM, MLPA) have been applied to detect CF mutations. Using different scanning procedures, we have identified over 250 *CFTR* mutations accounting for 97% of CF alleles. To evaluate this sensitivity, we have assessed the *CFTR* direct sequencing analysis in front of the scanning strategy. Ten samples with partial/total unknown genotype have been selected among our patients. Three out 20 alleles were already known (p.Phe508del, p.Cys592Tyr and c.1209G>A). Sequencing analysis showed one homozygous patient for the p.Ser549Arg mutation, one patient heterozygous for p.Met1101Lys and two patients bearing the p.Gly673X. Revision of our previous analysis has evidenced: (1) primers used in the early 1990s were unable to detect the p.Met1101Lys; (2) absence of heteroduplex bands in the two other mutations. It is well known the limitation of these techniques to detect homozygous changes; however, we do not find an explanation for the p.Gly673X mutation localized in the first half of exon 14. Usually, this large exon (723 nt) is analyzed in two overlapping fragments and other 23 different mutations have been identified in our series. Once again, we have taken advantage of the microsatellite haplotype (IVS8CA-IVS17bTA) associated to this mutation. Available CF samples still uncharacterized showing the 16–34 haplotype (n = 6) were sequenced specifically. The p.Gly673X mutation was identified in another patient. Our results evidence a higher sensitivity using sequencing analysis, a powerful tool now more affordable with the new equipments.

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4 Advances in the use of high resolution melting analysis for prenatal diagnosis and evaluation of p.Phe508del unlabelled probe

M. Poulou[1], A. Destouni[1,2], E. Kanavakis[1,2], M. Tzetis[1]. [1]*University of Athens, Medical Genetics, Athens, Greece;* [2]*Research Institute for the Study of Genetic and Malignant Disorders in Childhood, Athens, Greece*

High Resolution Melting Analysis (HRM) is an inexpensive, fast and robust method for detecting sequence changes in genomic DNA. The method consists of PCR amplification of the fragment in question, in presence of a dsDNA intercalation fluorescent dye. Following PCR the samples are melted revealing a unique melting profile according to their sequence composition. In our laboratory we evaluated the method using Idaho's Technology LightScanner® system and are currently using it as a routine method for screening. The primers used are from Montgomery *et al.* (*Clin. Chem.* 2007). All positive findings are confirmed by subsequent by-directional sequencing.

Factors which limit HRM's scanning and genotyping efficiency include primer and probe design as well as DNA quality and quantity. These factors hinder the broad application of HRM in samples coming from populations with genetic variability and in samples from various sources (already extracted DNA samples sent from different laboratories, old DNA samples or DNA from amniotic cells and/or CVS). In order to overcome the limitation arising from DNA quality and quantity we have developed a protocol which allows us to analyze consistently and with high specificity, samples from variable sources extracted in different ways.

In addition we focus on a specific drawback on the detection of the p.Phe508del mutation using the unlabelled probe as described in the paper by Montgomery *et al.* which could lead to misdiagnosis of carriers with the genotype p.[Leu467Phe;Met470Val;Phe508del] + [=] due to allele dropout caused by primer positioning.